

"According to one embodiment..." at page 2, line 20:

--Summary of the Invention

An attenuated strain of *Salmonella typhimurium* has been used as a vehicle for oral genetic immunization. Eukaryotic expression vectors containing the genes for b-galactosidase, or truncated forms of ActA and listeriolysin - two virulence factors of *Listeria monocytogenes*-that were controlled by an eukaryotic promoter have been used to transform a *S. typhimurium* aroA strain. Multiple or even single immunizations with these transformants induced a strong cytotoxic and helper T cell response as well as an excellent antibody response. Multiple immunizations with listeriolysin transformants protected the mice completely against a lethal challenge of *L. Monocytogenes*. Partial protection was already observed with a single dose. ActA appeared not to be a protective antigen.

C² The strength and the kinetics of the response suggested that the heterologous antigens were expressed within the eukaryotic host cells following transfer of plasmid DNA from the bacterial carrier strain. Transfer of plasmid DNA could be unequivocally shown *in vitro* using primary peritoneal macrophage. The demonstration of RNA splice products and expression of β -galactosidase in the presence of tetracycline - an inhibitor of bacterial protein synthesis - indicated that the gene was expressed by host cells rather than bacteria. Oral genetic immunization with *Salmonella* carriers provides a highly versatile system for antigen delivery, represents a potent system to identify candidate protective antigens for vaccination, and will permit efficacious generation of antibodies against virtually any DNA segment encoding an open reading frame.--

Please replace the paragraph starting at line 28 of page 2 with the following:

C³ --Further, the *Salmonella* strain according to the invention can be a *S. typhi* strain, especially *S. typhi* Ty21a.--

Please replace the paragraph starting at line 31 of page 2 with the following:

C⁴ --According to the invention *Salmonella* strains are comprised, wherein the eucaryotic

expression vector is or can be derived from the known plasmid pCMV β which comprises

-the structural gene of β -galactosidase (β -gal)

-under control of the human cytomegalovirus (CMV) immediate early promoter comprised by the plasmid pCMV β per se and includes

-a splice donor,

-two splice acceptor sites in between the promoter and the β -galactosidase gene, and facultatively

-the polyadenylation site of SV40.--

Please replace the paragraph starting at line 12 of page 3 with the following:

--According to the invention *Samonella* strains are comprised wherein the heterologous gene is selected from the group consisting of

- the *Esherichia coli*- β -galactosidase gene (LacZ-gene),

- a non-hemolytic truncated variant of the *Listeria monocytogenes*-listeriolysine gene (hly gene)

and

- a truncated variant of the *Listeria monocytogenes*-actA gene (actA gene).--

Please replace the heading "Legends to Figures" at line 3 of page 15 with

---Brief Description of the Drawings--.

Please replace the description of Fig. 1 on page 15, with the following:

--Fig. 1. Induction of cytotoxic T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Mice were

immunized either four times with two week intervals (A, B, D, E) or once (C, F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D-F) and spleen cells were restimulated once *in vitro* with a synthetic peptide comprising AA91-99 of listeriolysin (A-C) or with a mixture of purified ActA and hemolytically active listeriolysin which results in the class I presentation of ActA due to the pore-forming activity of listeriolysin (Darji et al., 1995a; Darji et al., 1997). Restimulated T cells were tested with radiolabelled P815 target cells at an effector to target ratio of 10:1. **Fig. 1A:** Specificity of the anti-listeriolysin cytotoxic response. Target cells were sensitized with henegglysozyme (HEL), peptide AA 91-99 of listeriolysin (pLLO) or control peptide of nucleoprotein of influenza virus (pNP). Displayed is the experiment with spleen cells from week 5 shown in panel B. Similar specificity was observed at all other time points. **Fig. 1B:** Kinetic of the cytotoxic response of mice immunized four times with pCMVhly. The arrows indicate the booster immunizations. **Fig. 1C:** Kinetic of the cytotoxic response of mice immunized once with pCMVhly. **Fig. 1D:** Specificity of the anti-ActA cytotoxic response. Target cells were sensitized with a mixture of Act A and listeriolysin (ActA + LLO), HEL, and listeriolysin (HEL + LLO) or listeriolysin alone (LLO). Displayed is the experiment with restimulated spleen cells from week 5 shown in panel E. Similar specificity was observed at other time points and including other synthetic peptides of various sources. **Fig. 1E:** Kinetic of the cytotoxic T cell response in mice immunized four times pCMVActA. Arrows indicate booster immunizations. **Fig. 1F:** Kinetic of the cytotoxic T cell response in mice immunized once with pCMVActA. The specificity of the cytotoxic response was further assessed by testing the spleen cells of mice immunized in a similar way with pCMV β (β -gal) on target cells sensitized with pLLO, ActA plus listeriolysin or a β -gal expressing transfectant of P815 (data not shown). Similarly, a specific cytotoxic T cell response was observed against β -gal, but the kinetic was not followed as systematically as for the two other antigens.--

Please replace the description of Fig. 2 starting on page 15, with the following:

--**Fig. 2.** Induction of cytotoxic T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Spleen (SPC) and lymph node cells (LNC) from the same mice tested for cytotoxic T cell responses displayed in Fig. 1 were tested for T helper responses. Mice were immunized either four times (A, B, C, E) or once (C, F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D, F) and restimulated

C⁸ *in vitro*. After two days proliferation was tested by incorporation of ³H-Thymidine. **Fig. 2A:** Specificity of the proliferative response of spleen cells from mice immunized with pCMVhly. T cells tested were the same as those displayed in panel B at week 11. Similar results were obtained at other time points. **Fig. 2B:** Kinetics of the proliferative response of spleen and lymph node cells from mice immunized four times with pCMVhly. Arrows indicate the booster immunizations. **Fig. 2C:** Kinetics of proliferative response of spleen and lymph node cells from mice immunized once with pCMVhly. **Fig. 2D:** Specificity of proliferative response of spleen cells from mice immunized four times with pCMAVActA. T cells tested were the same as those displayed in panel D at week 11. Similar results were obtained at other time points. **Fig. 2E:** Kinetics of the proliferative response of spleen and lymph node cells immunized four time with pCMVActA. Arrows indicate booster immunizations. **Fig. 2F:** Kinetics of the proliferative response of spleen and lymph node cells from mice immunized once with pCMVActA. Similarly, spleen and lymph node cells from mice immunized with pCMV β (β -gal) never reacted with either listeriolysin or ActA but could respond to restimulation with β -gal (data not shown).--

Please replace the description of Fig. 3 on page 16, with the following:

C⁹ --**Fig. 3.** Kinetics and subclass distribution of specific serum IgG from mice orally immunized with *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin, ActA or β -gal. Sera from the same mice tested for cytotoxic and proliferative T cell responses displayed in Figs. 1 and 2 were used and assayed in specific ELISA's. Mice were immunized four time (**Fig. 3A**) or once (**Fig. 3B**) with pCMVhly, pCMVActA or pCMV β , respectively, and pooled sera were tested for antigen specific serum IgG. To assess specificity, all sera were tested on all three antigens. Reactivity was only observed against the immunizing antigen (data not shown). Identical results were obtained by immunoblotting using the same antigens (data not shown). The subclass distribution 11 weeks after the first immunization was determined from the sera of individual mice immunized four time (closed symbols) or once (open symbols) with either pCMVhly (**Fig. 3C**) or pCMVActA (**Fig. 3D**).--

Please replace the description of Fig. 4 starting on page 16, with the following:

C¹⁰ --Fig. 4. Oral immunization with *S. typhimurium aroA* carrying the eukaryotic express plasmid which encodes listeriolysin induces a protective immune response, whereas immunization with bacteria carrying the expression plasmid for ActA is not protective. Groups of six mice were immunized four times with two week intervals (Fig. 4A) or only once (Fig. 4B) with *Salmonella* carrying pCMVhly, pCMVActA or pCMV β and challenged with a lethal dose of 5×10^4 *L. monocytogenes* EGD ($10 \times LD_{50}$) intravenously. Mice that had been immunized only once with pCMVhly became moribund after two days. However, four of them recovered and survived in good condition until the experiment was terminated two weeks later.--

Please replace the description of Fig. 5 on page 17, with the following:

C¹¹ --Fig. 5 Comparison of orally induced immune response elicited with *Salmonella* harboring prokaryotic or eukaryotic expression plasmids for β -gal. Mice were immunized with *Salmonella* harboring either the eukaryotic expression plasmid pCMV β or the plasmid pAH97 that constitutively expressed β -gal from the Pr and Ps promotor of *XylS* of *Pseudomonas putida*. Bacteria harboring the eukaryotic vector were administered orally once (\bullet), whereas bacteria express β -gal under the control of the prokaryotic promoter were administered either once (\blacklozenge) or four times with two week intervals (\blacktriangledown). The arrows indicate the time of booster immunizations. Fig. 5A: Cytotoxic response or restimulated spleen cells tested at an effector to target ratio of 10:1. The β -gal expressing transfectant P13.1 was used as target in the JAM assay. Fig. 5B. Proliferative helper T cell response of spleen cells with isolated β -gal as antigen. Fig. 5C: Antibody response against β -gal from pooled sera measured by ELISA. Data displayed in Fig. 5A-C were obtained with cells or sera from the same mice. All assays were performed as described in figures 1-3.--

Please replace the paragraph under the heading "ELISA" on page 22 with the following:

C12
--To evaluate the levels of immunoglobulins against LLO, Act-A and β -gal serum specimens, 96-well ELISA plates (Maxisorp, Nunc) were coated with 0.5 μ g/ml purified protein overnight at 4° C. Plates were washed three times with PBS/0.05% polyoxyethylene non-ionic detergent TWEEN 20 and then blocked with 3% BSA-PBS for 2 h at 37°C. Following two washes with PBS/0.05% polyoxyethylene non-ionic detergent TWEEN 20, serum samples if a 1:100 dilution were added to individual wells and incubated for 2-3 h at 37°C. Plates were washed above and biotinylated goat anti-mouse Ig (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. After three washes with PBS/0.05% polyoxyethylene non-ionic detergent TWEEN 20, horseradish peroxidase conjugated streptavidin (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. Plates were washed as above, developed with *o*-Phenylene diamine as substrate and measured in an ELISA reader at 490nm. For antigen specific IgG subclasses determination, peroxidase conjugated goat anti mouse IgG, IgG2A, IgG2b and IgG3 (Caltag laboratories, CA, USA) were used.--

Please replace the paragraph under the heading "RNA isolation and RT-PCR" on page 23 with the following:

C13
--In order to test for expression of β -gal transferred into the eukaryotic host cells via *Salmonella*, the mRNA was probed for the presence of splice products derived from the splice donor and acceptors of the expression plasmid. To this end, PECs were infected *in vitro* at an MOI of 10 with *S. typhimurium aroA* harboring the eukaryotic expression vector pCMB β and RNA was extracted as described (Chomczynski and Sacchi, 1987). RT-PCR of isolated RNA was performed. Briefly, 10 μ g of isolated total cellular RNA was resuspended in 20 μ l of DEPC-H₂O and incubated for 5 min at 70° C with 10 μ l of buffer containing 6 μ l of reverse transcriptase buffer (250 mM Tris-HCl, 375 mM KCl, 15mM MgCL₂); 0,4mM dNTPs; 0,05 U random hexamers (Pharmacia, Uppsala, Sweden); and 1 mM DTT. Samples were spun down for 2 min at 15,000 rpm and 40 U RNasin ribonuclease inhibitor (Promega) together with 200 U Superscript reverse transcriptase (Gibco, BRL) were added. RNA was reverse transcribed for 445 min at 37°C and the reaction was stopped by heating the samples at 95°C for 1 min followed by a short incubation on ice. Subsequently, 500 ng of cDNA product was amplified by PCR in a